PB 279 183

PUBLICATION

129

Copyright, 1964, by the Society for Experimental Biology and Medicine.
Reprinted from PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE, 1964, v115, 487-490

TOXICOLOGY PROGRAM
Technology Branch
Communicable Disease Center
Atlanta 22, Ga.

Effects of Dieldrin or DDT *in vivo* on Alpha-Alanine, Gamma-Aminobutyrate, Glutamine, and Glutamate in Rat Brain. (28948)

ROBERT F. WITTER AND WILLIAM L. FARRIOR, JR.* (Introduced by G. R. Cooper)
Toxicology Section, Technology Branch, Communicable Disease Center, Public Health Service, U. S. Department of HEW, Atlanta, Ga.

The biochemical mechanism by which mammals are poisoned with chlorinated hydrocarbon insecticides, such as 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-endo, exo-5,8-dimethano-naphthalene (dieldrin) and 2,2-bis-(p-chlorophenyl)-1,1,1-trichloroethane (DDT), is unknown. Both of these insecticides produce convulsions in man (1-4) and experimental animals(5,6). The convulsions produced by dieldrin closely resemble the *grand mal* seizure of epilepsy. They occur without warning, are intermittent, and are clonicotonic in nature(3,4,6). On the other hand, animals exposed to a lethal dose of DDT show gradually increasing muscular tremor, which may last several hours and lead to convulsions that may be terminal(5). Symptoms observed in man have been similar to those noted in experimental animals(1,2). The marked difference in symptomatology between the two insecticides suggests a difference in their modes of action. Under these circumstances, any change noted with one substance and not with the other might reveal some clue as to a specific mode of action.

The free amino acid level of the brain is considerably higher than that of plasma and the pattern of the free amino acid pool is different from that of cerebral protein(7). A number of workers have investigated the effect of various convulsants on the levels of amino acids in brain, particularly the levels of glutamic acid, glutamine, and γ-aminobutyric acid(8-25). Further, it has been suggested that γ-aminobutyric acid may be a depressant neurohormone which may oppose the excitatory action of acetylcholine(26). As far as we are aware, no reports have appeared in the literature on the effects of dieldrin or DDT on these substances.

Therefore, a study was made of the levels of these amino acids in the brains of rats poisoned with DDT or dieldrin in an attempt to obtain some insight concerning the mode of action of these chlorinated compounds. In these experiments, the animals were dosed with large amounts of the toxicant to obtain severe poisoning. They were sacrificed when exhibiting pronounced symptoms but before moribund changes could begin.

Experimental section. Individually caged female albino rats of the Sherman strain, 4 to 4½ months of age and weighing 172-262 g, were used in all experiments. The insecticides were administered in peanut oil by stomach tube at the rate of 5 ml/kg body wt (160 mg/kg of dieldrin or 400 mg/kg of DDT). These dosage levels are approximately 4 times the oral LD_{50} dose of each insecticide(27). Control groups received peanut oil alone.

In preliminary experiments it was found that animals given dieldrin at this level survived 4 convulsions and died within 114 to 176 minutes after dosing, averaging 146 ± 23 (Standard Deviation) minutes. On the basis of these experiments, it was decided to sacrifice the animals during their fourth convulsion. Therefore, the dieldrin-poisoned animals were sacrificed within 72 to 109 minutes after dosing, averaging 89 ± 14 minutes. Onset of the first convulsion in the group occurred within 36 to 81 minutes after dosing, averaging 53 ± 15 minutes. In the case of the animals given DDT, preliminary experiments showed that death occurred within 200 to 470 minutes, averaging 394 ± 109 minutes. On the basis of these results, it was decided to sacrifice the DDT-poisoned animals after 120 minutes of visible body tremor. The onset of tremor was defined as a condition in which the animal's back was arched and the entire body was in tremor. This occurred within 100 to 180 minutes

* Present address: U. S. Dept. of Interior, Bureau of Mines, Collins Ferry Road, Morgantown, W. Va.

4

after dosing, averaging 131 ± 23 minutes.

Control animals were sacrificed at the same time as corresponding rats in the experimental group. The rats were decapitated and the heads were dropped into liquid nitrogen within 4 seconds; the whole brain then was extracted essentially according to the procedure of Roberts and Frankel(28).

Amounts of extract equivalent to 20 mg of brain, fresh weight basis, were applied as 20 μl bands on Whatman No. 1 paper for the analysis of γ-aminobutyric acid, glutamic acid, and glutamine. Glutamic acid and γ-aminobutyric acid were separated at 25°C in phenol saturated with water and containing 0.025% 8-hydroxyquinoline. O-Phosphoryl ethanolamine runs in the same position as glutamic acid in this solvent but the amount present is so small, in relation to the large amount of dicarboxylic acid(29), that the interference in subsequent analysis was neglected. Glutamine was separated in a solvent containing 2-butanol—95% ethanol—6% ammonium hydroxide (100:25:35 v/v), as described by Roland and Gross(30). The ammonium hydroxide was prepared from concentrated ammonium hydroxide by diluting with 4 volumes of water. The mobility of glutamine in this solvent has not been described. After 72 hours of development at 25°C, this amide was found to run between the aspartic-glutamic acid and the serine-glycine-histidine spots, and its movement in relation to valine was 0.32 as compared with 0.12 for glutamic acid and 0.42-0.43 for serine-glycine-histidine. Recovery studies showed that the rate of hydrolysis of glutamine in this solvent was negligible. The quantity of amino acids present was determined from comparison with standards chromatographed at the same time, essentially according to the method described by Roberts and Frankel (31).

Alanine was separated by 2-dimensional chromatography. Twenty μl spots, equivalent to 40 mg of brain, fresh weight basis, were applied to 46 × 57 cm Whatman No. 1 filter paper, then chromatographed at 25°C in phenol-water followed by collidine-lutidine (1:1 v/v) saturated with water(32). It was not feasible to run more than 8 two-dimensional chromatograms at a time. Therefore, extracts of the brains of 4 control and 4 experimental animals were run simultaneously without standards as described in Table II, and the results reported as optical density.

Studies were also conducted to prove that the spots analyzed did, indeed, contain the amino acids, γ-aminobutyric acid, glutamic acid, glutamine, or alanine. Pooled brain extracts, before and after acid hydrolysis, were co-chromatographed with standards and with internal standards in the phenol-water solvent followed by the collidine-lutidine solvent or in the phenol-water system followed by 2-butanol-ethanol-ammonia. The spots of the unknowns and the standards run to the same position and the internal standards were recovered quantitatively. Further proof that the spot corresponding to glutamine on 2-butanol-ethanol-ammonia chromatograms contained only glutamine was obtained by chromatography of the eluate of this spot in the previously-mentioned 2-dimensional system. No ninhydrin-reacting constituent other than glutamine was found. Also, glutamic acid was the only ninhydrin-reacting substance found after acid hydrolysis of this eluate and subsequent chromatography in the same 2-dimensional systems.

Results. The effects of dieldrin and DDT on levels of gamma-aminobutyric acid, glutamine, glutamic acid, and alanine are illustrated in Tables I and II. As shown in the Tables, DDT had no significant effect on levels of gamma-aminobutyric acid, glutamic acid, alanine or glutamine. On the other hand, an increase of 24% was noted in the level of gamma-aminobutyric acid after administration of dieldrin. This increase was significant at level $p = 0.02$ to 0.01. This result is in agreement with recent studies(15, 18,23,33) that have shown that the occurrence of seizures is not necessarily related to decreased levels of gamma-aminobutyric acid in the whole brain. The present results do not rule out the possibility of localized decreases in the content of this amino acid that are not reflected in the concentration of gamma-aminobutyric acid in the whole brain.

However, the most striking change, easily visible on the chromatograms, was an aver-

TABLE I. Effects of Dieldrin or DDT on Concentration of Glutamine, Gamma-Aminobutyric Acid, and Glutamic Acid in Rat Brain. Nine control and 9 experimental animals were employed in studies with dieldrin; 8 control and 8 experimental animals in DDT experiments. Differences between control and experimental groups were tested for statistical significance by "Student's" T-test(37).

		Control group, mg/g brain			Experimental group, mg/g brain		
Toxicant		Glu NH_2	γ-ABA	Glu A	Glu NH_2	γ-ABA	Glu A
Dieldrin	Range	.33–.80	.14–.21	1.00–1.73	.32–1.00	.15–.26	.85–1.57
	Mean	.52	.17	1.32	.62	.21	1.22
	S.D.	.15	.027	.25	.21	.035	.25
	P				.3–.2	.02–.01	.5–.4
DDT	Range	.40–.56	.12–.19	1.00–1.53	.23–.79	.09–.21	.73–1.50
	Mean	.48	.16	1.25	.56	.16	1.15
	S.D.	.065	.022	.18	.19	.041	.32
	P				.3–.2	.8–.7	.6–.5

TABLE II. Effect of Dieldrin and DDT on Relative Alanine Content of Rat Brain.

Chromatographic run		Dieldrin experiment, optical density at 570 mμ		Chromatographic run	DDT experiment, optical density at 570 mμ	
		Controls	Dieldrin		Controls	DDT
1	Range	.105–.184	.190–.315	3	.070–.095	.070–.125
	Mean	.137	.248		.081	.090
	S.D.	.034	.053		.036	.024
	P		.02 –.01			.7 –.6
2	Range	.095–.115	1.50 –.225			
	Mean	.096	.189			
	S.D.	.013	.030			
	P		<.001			
1 and 2	P		.01 –.001			

Extracts of brains of 4 animals in control and dieldrin groups, respectively, were chosen at random and analyzed in each of the 2 chromatographic runs (Nos. 1 and 2) so that a total of 8 animals out of 9 in the respective groups of Table I were studied. Extracts of brains of 4 animals in control and DDT-poisoned groups, respectively, in Table I were selected at random and analyzed by 2-dimentional chromatography in Run No. 3. Differences between control and experimental groups in a given chromatographic run were tested for significance by "Student's" T-distribution as modified by Fisher(37) using logarithms of optical densities of the extracts. In the case of dieldrin in which 2 chromatographic runs were made, an additional test for statistical significance of the differences between experimental and control groups using the data obtained from both chromatographic runs was carried out by a modified "T" test.

$$t = \frac{E_1 - C_1 + E_2 - C_2}{\sqrt{\frac{ms^2}{n}}} \qquad S^2 = \frac{\Sigma(e_1 - E_1)^2 + \Sigma(c_1 - C_1)^2 + \Sigma(e_2 - E_2)^2 + \Sigma(c_2 - C_2)^2}{(n-1)m}$$

Where n = No. of determinations per mean; m = No. of means; e_1, and c_1 are the logarithms of optical densities on a given day of the samples in experimental and control groups, respectively. E_1 and C_1 are the means of the logarithms of optical densities of the samples in control and experimental groups on a given day. This equation was developed by Dr. Richard G. Cornell, CDC Statistics Section, and can be derived from the equation on page 85 of Snedecor(38).

age rise of 80% in concentration of alanine in the brains of rats given dieldrin. This was statistically significant at the level of $p = <0.01$. The rise in concentration of this amino acid is probably the result of the inhibition by dieldrin of the oxidations of the Krebs tricarboxylic acid cycle in the brain, possibly at the α-ketoglutarate stage. The facts that DDT does not inhibit the respiration of mammalian brain(34,35) and does not change the level of alanine, glutamine, glutamic acid or γ-aminobutyric acid are both

consistent with this hypothesis as to the action of dieldrin. However, a direct test of the effects of dieldrin upon the ability of rat brain to oxidize members of the Krebs cycle other than succinate(36) has not been carried out.

Summary. Studies were made of the effects of lethal doses of dieldrin or DDT on the free amino acid pattern of the brains of rats exhibiting severe symptoms of poisoning. Elevated levels of α-alanine and γ-aminobutyrate were noted with dieldrin but not with DDT, indicating a different mode of action of the 2 insecticides.

1. Garrett, R. M., *J. Med. Assn. State Alabama*, 1947, v17, 74.
2. Velbinger, H. H., *Pharmazie*, 1947, v2, 268.
3. Hayes, W. J., Jr., *Public Health Rep.*, 1957, v72, 1087.
4. ———, *Bull. World Health Org.*, 1959, v20, 891.
5. Domenjoz, R., *Arch. Exp. Path. and Pharm.*, 1948, v208, 144.
6. Hayes, W. J., Jr., Ferguson, F. F., Cass, J. S., *Am. J. Trop. Med.*, 1951, v31, 519.
7. McIlwain, H., in *Biochemistry and the Central Nervous System*, 2nd Ed., Little, Brown and Co., Boston, 1959, p106.
8. Cravioto, R. O., Massieu, G., Izquierdo, J. J., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 856.
9. Dawson, R. M. C., *Biochem. Biophys. Acta*, 1953, v11, 548.
10. Gammon, G. D., Gumnit, R., *Trans. Am. Neurol. Assn.*, 1957, v82, 57.
11. Killam, K. F., Bain, J. A., *J. Pharm. and Exp. Ther.*, 1957, v119, 255.
12. Roberts, E., Lowe, I. P., Guth, L., Jelinek, B., *J. Exp. Zool.*, 1958, v138, 313.
13. Baxter, C. F., Roberts, E., PROC. SOC. EXP. BIOL. AND MED., 1959, v101, 811.
14. Häkkinen, H. M., Kulonen, E., *Nature*, 1959, v184, 726.
15. Kessel, D., *Fed. Proc.*, 1959, v18, 258, A-1020.
16. Rindi, G., Ferrari, G., *Nature*, 1959, v183, 608.
17. Ernsting, M. J. E., Kafoe, W. F., Nauta, W. Th., Oosterhuis, H. K., de Waart, C., *J. Neurochem.*, 1960, v5, 121.
18. McKhann, G. M., Albers, R. W., Sokoloff, L., Mickelsen, O., Tower, D. B., in *Inhibition in the Nervous System and Gamma-Aminobutyric Acid*, Pergamon Press, New York, 1960, p169.
19. Purpura, D. P., Berl, S., Gonzalez-Monteagudo, O., Wyatt, A., *ibid.*, 1960, p331.
20. Tursky, T., *Nature*, 1960, v187, 323.
21. Ferrari, R. A., Arnold, A., *Biochim. Biophys. Acta*, 1961, v52, 361.
22. Higgins, E. S., *Biochem. Pharm.*, 1962, v11, 394.
23. Massieu, G. H., Tapia, R., Ortega, B. G., *Biochem. Pharmacol.*, 1962, v11, 976.
24. Massieu, G. H., Ortega, B. G., Syrquin, A., Tuena, M., *J. Neurochem.*, 1962, v9, 143.
25. Tursky, T., Sajter, V., *ibid.*, 1962, v9, 519.
26. Roberts, E., Rothstein, M., Baxter, C. F., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 796.
27. Gaines, T. B., *Toxicol. Appl. Pharmacol.*, 1960, v2, 88.
28. Roberts, E., Frankel, S., *Cancer Res.*, 1949, v9, 645.
29. Ansell, G. B., Richter, D., *Biochem. J.*, 1954, v57, 70.
30. Roland, J. F., Jr., Gross, A. M., *Anal. Chem.*, 1954, v26, 502.
31. Roberts, E., Frankel, S., *J. Biol. Chem.*, 1950, v187, 55.
32. Dent, C. E., *Biochem. J.*, 1948, v43, 169.
33. Baxter, C. F., Roberts, E., PROC. SOC. EXP. BIOL. AND MED., 1960, v104, 426.
34. Jandorf, B. J., Sarett, H. P., Bodansky, O., *J. Pharmacol. and Exp. Ther.*, 1946, v88, 333.
35. Judah, J. D., *Brit. J. Pharmacol.*, 1949, v4, 120.
36. Hosein, E. A., Proulx, P., *J. Agr. Food Chem.*, 1960, v8, 428.
37. Fisher, R. A., *Proc. of Internat. Mathematical Congress*, Toronto, 1924, 805.
38. Snedecor, G. W., *Statistical Methods*, 5th Ed., Iowa State Coll. Press, Ames, 1956, p85.

Received October 21, 1963. P.S.E.B.M., 1964, v115.